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Microbial ecology of arsenic-mobilizing Cambodian sediments: lithological controls uncovered by stable-isotope probing

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Summary

Microbially mediated arsenic release from Holocene and Pleistocene Cambodian aquifer sediments was investigated using microcosm experiments and substrate amendments. In the Holocene sediment, the metabolically active bacteria, including arsenate-respiring bacteria, were determined by DNA stable-isotope probing. After incubation with ^{13}C-acetate and ^{13}C-lactate, active bacterial community in the Holocene sediment was dominated by different *Geobacter* spp.-related 16S rRNA sequences. Substrate addition also resulted in the enrichment of sequences related to the arsenate-respiring *Sulfurospirillum* spp. ^{13}C-acetate selected for ArrA related to *Geobacter* spp. whereas ^{13}C-lactate selected for ArrA which were not closely related to any cultivated organism. Incubation of the Pleistocene sediment with lactate favoured a 16S rRNA-phylotype related to the sulphate-reducing *Desulfovibrio oxamicus* DSM1925, whereas the ArrA sequences clustered with environmental sequences distinct from those identified in the Holocene sediment. Whereas limited As(III) release was observed in Pleistocene sediment after lactate addition, no arsenic mobilization occurred from Holocene sediments, probably because of the initial reduced state of As, as determined by X-ray Absorption Near Edge Structure. Our findings demonstrate that in the presence of reactive organic carbon, As(III) mobilization can occur in Pleistocene sediments, having implications for future strategies that aim to reduce arsenic contamination in drinking waters by using aquifers containing Pleistocene sediments.

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Introduction

In South and South East Asia, where high concentrations of arsenic in groundwater are causing a humanitarian disaster (Smith *et al.*, 2000), aquifer sediments have been studied largely in terms of their geological, mineralogical and geochemical characteristics (Acharyya *et al.*, 2000; Berg *et al.*, 2001; 2007; Polya *et al.*, 2003; Ravenscroft *et al.*, 2005; 2009; Zheng *et al.*, 2005; Charlet and Polya, 2006; Buschmann *et al.*, 2007; Rowland *et al.*, 2008). However, studies based on arsenic-rich South East Asian sediments support the consensus that the oxidation of organics coupled to the reduction of As(V) sorbed to mineral surfaces plays a major role in As(III) mobilization in anoxic aquifers (e.g. van Geen *et al.*, 2004; Islam *et al.*, 2004; Héry *et al.*, 2010). Metal-reducing bacteria conserve energy by using metals including Fe(III) and the metalloid As(V) (arsenate) as electron acceptors. Respiratory arsenate reduction is catalysed by an enzyme of which the *arrA* gene is a key component, and known As(V)-respiring bacteria are affiliated with many different bacterial genera (Oremland and Stolz, 2005). Profiling of the bacterial structure based on 16S ribosomal RNA (rRNA) gene phylogeny and the study of arsenate reductase genes in arsenic-rich sediments have emphasized the enrichment of metal-reducing *Geobacter* spp. (Islam *et al.*, 2004; Lear *et al.*, 2007; Rowland *et al.*, 2007), which have the potential to respire both Fe(III) and As(V) (Lloyd *et al.*, 2011). Furthermore, the probable influence of *Geobacter* in arsenic biogeochemistry in groundwater

has been highlighted recently by a transcriptomic approach (Giloteaux *et al*., 2013).

The use of ^{13}C-labelled organic substrates and DNA stable-isotope probing (DNA-SIP) can reveal the active members of the microbial community, and it has been used to study a wide range of microbial processes in various environments (reviews by Neufeld *et al*., 2007a; Uhlík *et al*., 2009). However, for arsenic-rich sediments, DNA-SIP has only been used once to characterize ^{13}C-acetate utilizing arsenate-respiring bacteria (Lear *et al*., 2007). Furthermore, the majority of microcosm studies with arsenic-rich sediments have been carried out using acetate as the organic carbon proxy to enhance microbial activity (e.g. van Geen *et al*., 2004; Islam *et al*., 2004; Gault *et al*., 2005; Héry *et al*., 2010). Thus, it is not clear whether incubation with different organic substrates can affect the structure of the microbial community and its potential for As(III) mobilization.

Most previous microbial ecology studies of arsenic-rich sediments have focused on sediments collected from Holocene aquifers, mainly because Pleistocene aquifers are generally thought to be less affected by arsenic contamination (Acharyya *et al*., 2000; Ravenscroft *et al*., 2005; Zheng *et al*., 2005) and are also more difficult to access. The use of waters from deeper Pleistocene aquifers (well switching) may represent a viable strategy for limiting arsenic uptake by at-risk populations, as shown in Bengal (van Geen *et al*., 2003; Polya and Charlet, 2009; Burgess *et al*., 2010). Recent studies have suggested that biogeochemical mobilization of As(III) may occur to a lesser extent in Pleistocene sediments (Sutton *et al*., 2009; Al Lawati *et al*., 2012). Another study showed that the addition of arsenate respiring *Shewanella* spp. to Pleistocene sediments is necessary to trigger the release of As(III) (Dhar *et al*., 2011). However, the actual potential for indigenous microbial communities to mobilize As(III) under anoxic conditions, when stimulated by a simple organic substrate that can act as electron donor, needs to be explored further. Therefore, in this study the structure of the whole bacterial community and of the As(V)-respiring bacteria were determined using culture-independent techniques in two arsenic-rich Cambodian sediments (i.e. containing higher As content than the crustal average): a Holocene and Pleistocene sediment with contrasting mineralogical and geochemical characteristics, depositional history, and organic matter composition and abundance. In addition, the Holocene sediment was incubated with both ^{13}C-acetate and ^{13}C-lactate, to investigate and compare the impact of two different short-chain organic acids on the microbial community structure and reductive As(III) mobilization. The use of ^{13}C-labelled substrates combined with DNA-SIP facilitated the profiling of the active members of the bacterial community.

The aims of this study were (i) to characterize and compare the bacterial community-metabolizing acetate or lactate and the As(V)-respiring bacteria in a Holocene and a Pleistocene sediment, (ii) to investigate the effect of different organic carbon amendments on bacterial community structure and As(III) mobilization, and (iii) to investigate the potential for As(III) mobilization in Holocene and Pleistocene sediments. A better understanding of the biotic and abiotic factors controlling arsenic mobilization and immobilization in South East Asian aquifers, including the lithological characteristics of the sediments, is ultimately required to provide insight into appropriate strategies to reduce the exposure to arsenic through the consumption of contaminated groundwater.

Results

Sediment characteristics

Two sediments with distinct characteristics were collected from an arsenic rich Cambodian aquifer: Holocene sediments were composed of a mix of clay and fine-grained grey sand whereas Pleistocene sediments were composed of fine to medium orange sand. Our further analyses indicated clear differences between these two sediments with regard to their arsenic oxidation state and organic content. The dominant arsenic species [as determined by X-ray Absorption Near Edge Structure (XANES)] in the Pleistocene sediment was As(V) (100%), whereas in the Holocene sediment arsenic was mainly present in the reduced As(III) state (85%), with As(V) (7%) and As_2S_3 (8%) also present (Table 1). Furthermore, the total carbon (TC), total organic carbon (TOC) and total nitrogen (TN) content (1.48%, 1.21% and 0.25% respectively) was markedly higher in the Holocene than in the Pleistocene sediment (TC = 0.28%, TOC = 0.23% and TN

Table 1. Arsenic speciation in the solid phase (based on X-ray Absorption Near Edge Structure spectra) of Holocene sediments and of Pleistocene sediments before and after incubations with lactate.

		% arsenic in form of			
Sample	Incubation period (d)	Arsenate	Arsenite	As_2S_3	Fit index %
Holocene sediment	–	7	85	8	4.37
Pleistocene sediment	–	100	–	–	5.01
P + lactate	77	96	4	–	2.44

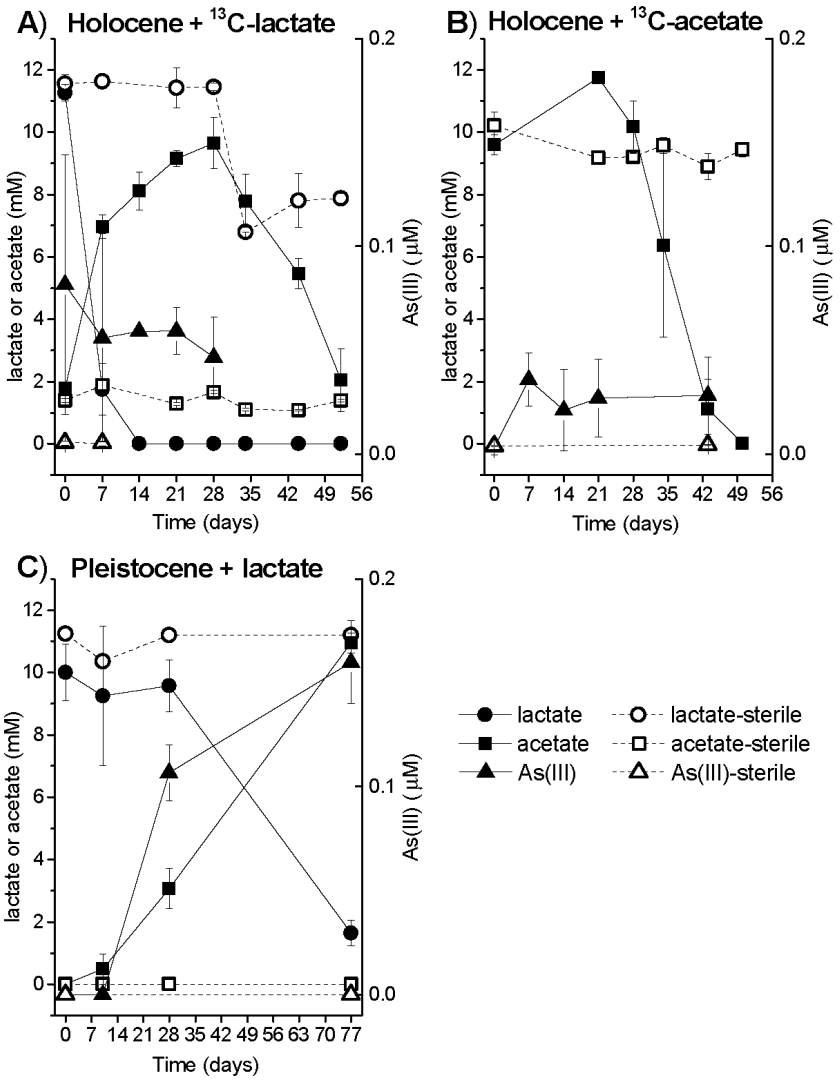

Fig. 1. The concentration of lactate (circles), acetate (squares) and mobilized As(III) (triangles) during the incubation of the (A) Holocene sediment + ^{13}C-lactate, (B) Holocene sediment + ^{13}C-acetate and (C) Pleistocene sediment + lactate microcosms. Black symbols indicate biotic microcosms, and white symbols indicate sterile (abiotic) microcosms.

below detection limits). Detailed analysis of the organic biomarker distribution, which further highlights the large differences between the two sediments, is provided in Supporting Information Appendix S1.

Substrate utilization and As(III) release during microcosm incubations

Anaerobic microcosms were set up with Holocene (H) sediment amended with 10 mM ^{13}C-lactate or 10 mM ^{13}C-acetate. After incubation under anaerobic conditions, a clear succession of substrate utilization was observed. In the 'H + ^{13}C-lactate' microcosms, the largest proportion of the lactate (84%) was degraded rapidly within 7 days (Fig. 1A), and the degradation of lactate was followed by an increase in the concentration of acetate up to 9.6 mM on day 28, before it was largely degraded by day 52 (Fig. 1A). The concentration of acetate in the 'H + ^{13}C-acetate' treatment mostly decreased between days 28 and 43 (Fig. 1B). In the microcosms set up with the Pleistocene sediment amended with 10 mM lactate, lactate utilization within the first 28 days of incubation was accompanied with the production of 3 mM acetate (Fig. 1C). By the end of the incubation period (77 days), most of the lactate was degraded (84%), while acetate had accumulated in the microcosms (11 mM; Fig. 1C). Contrary to lactate, acetate was not degraded in the Pleistocene sediment over the time period used in this study (data not shown). In all abiotic controls (sterilized by autoclaving), no significant degradation of the added substrates (lactate or acetate) or formation of acetate was observed during the incubation period (Fig. 1A–C), indicating that utilization of these organic substrates was microbially driven.

In the microcosms set up with Holocene sediment and amended with ^{13}C-lactate or ^{13}C-acetate (Fig. 1A and 1B respectively), As(III) release was not observed during the incubation period. Thus, arsenic speciation in the solid phase of these incubations was not examined. In contrast,

low As(III) release occurred in the aqueous phase of the 'P + lactate' microcosms (up to 0.16 μM/12 ppb; Fig. 1C), and it was accompanied by a small increase in the As(III) content in the solid phase (4%; Table 1).

Phylogenetic structure of bacterial communities

In the 16S rRNA gene clone library constructed from the Holocene sediment (73 clones), 21 operational taxonomic units (OTUs) were identified at 97% similarity level, with none of the genera representing more than 20% of the sequences. The bacterial community of the Holocene sediment was dominated by β-*Proteobacteria* (60%), *Firmicutes* (21%) and δ-*Proteobacteria* (8%) phyla, with most of the sequences affiliated to the *Hydrogenophaga* (29%), *Acidovorax* (16%), *Acetobacterium* (8%) and *Geobacter* (8%) genera (Fig. 2). In addition, one sequence was closely related (99% similarity) to the dissimilatory arsenate-reducing strain *Sulfurospirillum halorespirans* PCE-M2 (GenBank accession number: AF218076) (Luijten *et al*., 2003).

After incubations with ^{13}C-labelled substrates, DNA-SIP fractionation resulted in the separation of the heavy and light DNA fractions, evident by the distinct denaturing gradient gel electrophoresis (DGGE) profiles produced by the different DNA fractions (Fig. 3). It was therefore possible to identify the active members of the microbial communities in these microcosms by 16S rRNA gene cloning and sequencing of the heavy (^{13}C-labelled) DNA fraction. After incubation with ^{13}C-lactate, most of the sequences in the heavy DNA fraction belonged to the *Geobacter* (71% of the sequences), *Sulfurospirillum* (15%) and

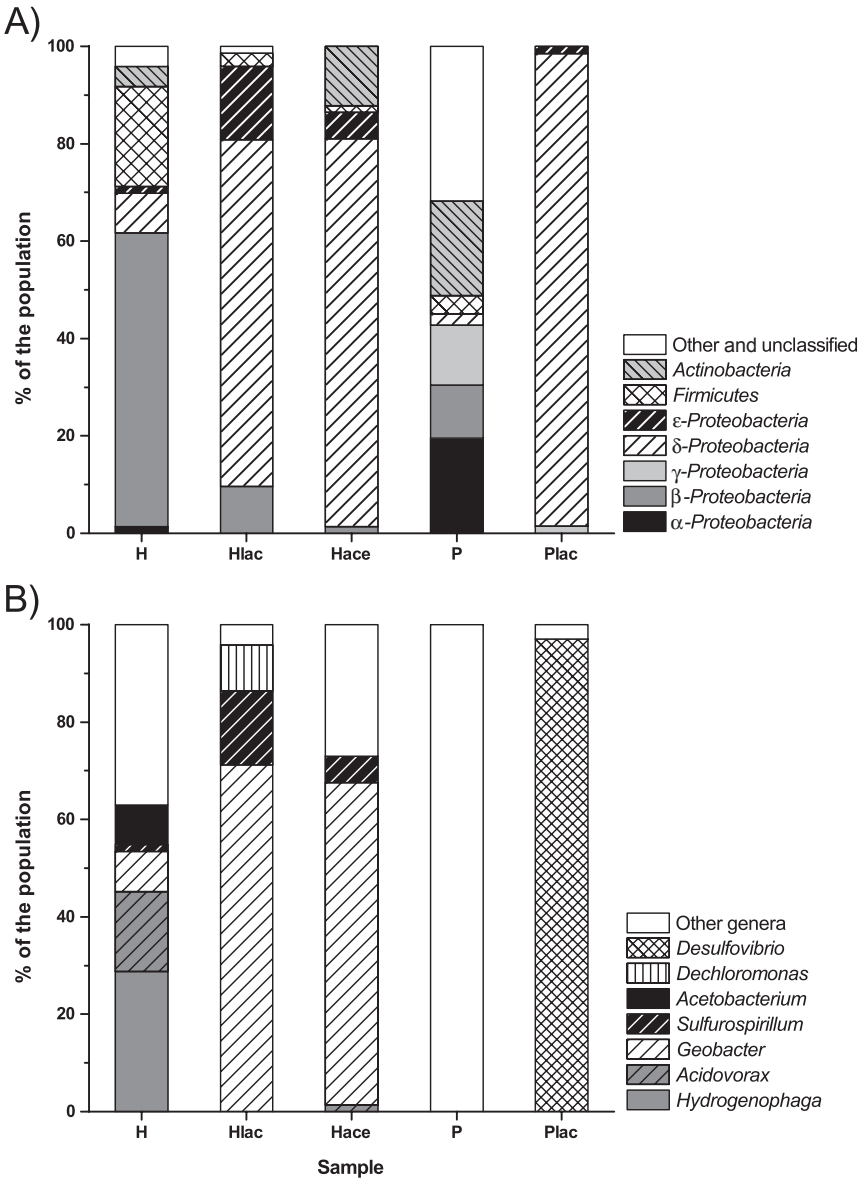

Fig. 2. Phylogenetic comparison at the (A) phylum/class and (B) genus level based on bacterial 16S rRNA gene sequencing in Holocene (H) and Pleistocene (P) sediments, the heavy DNA fractions of the 'Holocene sediment + ^{13}C-lactate' (Hlac) and 'Holocene sediment + ^{13}C-acetate' (Hace) incubations, and the lactate-amended Pleistocene sediment (Plac). Details about the treatments are shown in Table 2. Only genera representing more than 1.3% of the total number of clones (seven sequences and above) are shown.

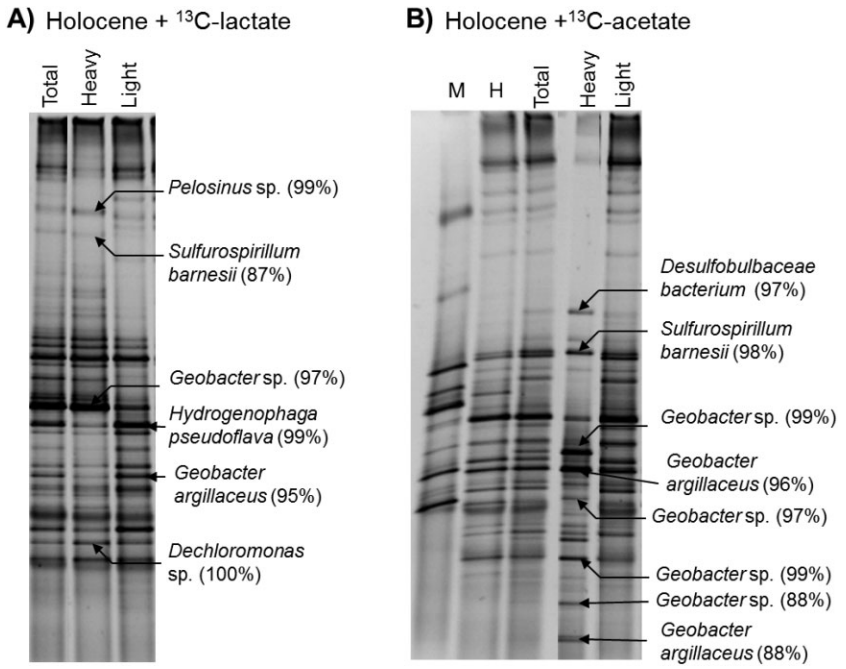

Fig. 3. DGGE of bacterial communities in Holocene sediments after incubation with (A) ^{13}C-lactate and (B) ^{13}C-acetate, following DNA-SIP fractionation. H = Holocene sediment (prior to incubations); Total = total DNA (after incubations, but prior to DNA-SIP fractionation); Heavy = heavy DNA fraction; Light = light DNA fraction; M = DGGE marker. Selected DGGE bands were excised (as shown) and sequenced; sequence similarity with the closest cultivated organism is indicated.

Dechloromonas (10%) genera of the δ-, ε- and β-*Proteobacteria* respectively (Fig. 2). Similarly, most of the sequences in the heavy DNA fraction of the 'H + ^{13}C-acetate' treatment were affiliated to the *Geobacter* (66%) and *Sulfurospirillum* (5%) genera. However, phylogenetic analysis of the *Geobacter*-related 16S rRNA gene sequences in this study showed that distinct *Geobacter* phylotypes were selected depending on the treatment (Fig. 4). In the Holocene sediment prior to incubation, most (five out of six) *Geobacter* 16S rRNA gene sequences were closely related (99% ID similarity) to environmental sequences from West Bengal As(III) mobilizing sediments (Héry *et al*., 2010), and one sequence was related to *Geobacter pickeringii* G13 (DQ145535). Incubation with ^{13}C-lactate led to the dominance of sequences closely related (98% ID similarity) to *Geobacter chapelleii* 172 (U41561) and *Pelobacter propionicus* DSM 2379 (CP000482). In contrast, incubation with ^{13}C-acetate led to the enrichment of a phylotype (31% of the population) related to *G. pickeringii* G13 (DQ145535) and another phylotype (26% of the population), which shared 98% similarity with *P. propionicus* DSM 2379 (CP000482) and 97% ID similarity with many *Geobacter* spp., including *Geobacter uraniireducens* Rf4 (EF527427), *G. chapelleii* 172 (U41561) and *Geobacter toluenoxydans* TMJ1 (EU711072) (Fig. 4). The sequenced DGGE bands following DNA-SIP fractionation (Fig. 3) were largely in agreement with the results from the 16S rRNA gene clone libraries, as the dominant bands in the heavy DNA fractions of the 'H + ^{13}C-lactate' treatment belonged to the *Geobacter*, *Sulfurospirillum* and *Dechloromonas* genera, and those of the 'H + ^{13}C-acetate' treatment were affiliated to the *Geobacter* and *Sulfurospirillum* genera.

The Pleistocene sediment was characterized by a complex bacterial community, as 61 OTUs were identified (out of 82 sequences, at 97% OTU similarity level), with none of them representing more than 7% of the sequences. Despite the high number of OTUs present, there was no clear overlap between the sequences of the Holocene and the Pleistocene sediments. The dominant bacterial phyla were *Actinobacteria* and α-*Proteobacteria* (20% of the sequences each), with β-, γ-, δ-*Proteobacteria*, *Acidobacteria* and *Firmicutes* also present (Fig. 2). Bacteria detected in this sample included those affiliated with the following taxa: *Propionivibrio* (five sequences), *Aeromonas* (five), *Acidobacteria Gp6* (four) and *Gp21* (two), *Pelagibius* (two) and *Enterobacter* (two). The following were also detected (one sequence each): *Pseudomonas*, *Sphingomonas*, *Pseudolabrys*, *Methylobacterium*, *Microvirga*, *Delftia*, *Tumebacillus*, *Euzebya* and *Dietzia*. The existence of sequences closely related (99% and 98% ID similarity respectively) to the obligate aerobe *Methylobacterium oxalidis* (AB607860) and to the strictly anaerobe *Propionivibrio dicarboxylicus* CreMal1 (NR_026477) indicates that the Pleistocene sediment may have consisted of different micro-niches, which were inhabited by both aerobic and anaerobic organisms. After addition of lactate to the Pleistocene sediment and incubation for 77 days ('P + lactate' treatment), 97% (66 out of 68) of the sequences were closely related (99% ID similarity) to the sulphate- andnitrate-reducing δ-*Proteobacterium Desulfovibrio oxamicus* DSM 1925 (DQ122124).

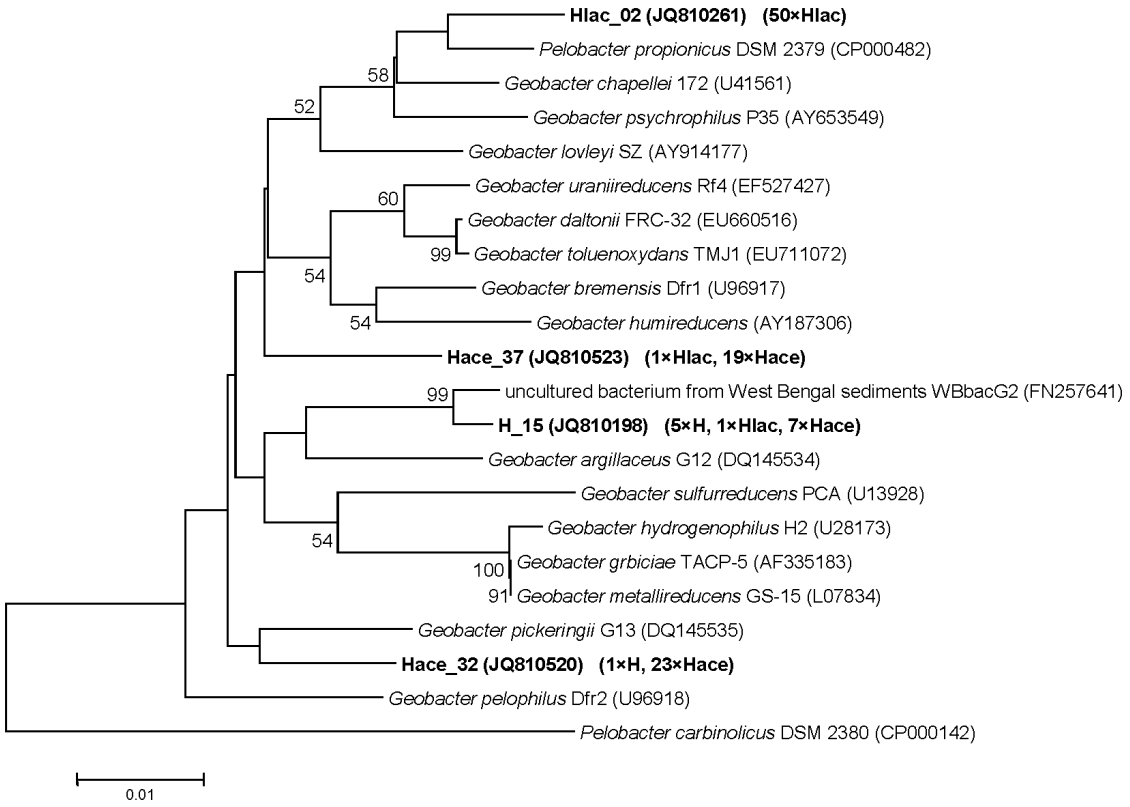

Fig. 4. Phylogenetic tree of *Geobacter*-related 16S rRNA gene sequences of this study and selected *Geobacter* species. The number of clones contained in each corresponding OTU (based on a 97% cut-off) is indicated in brackets (the same OTU can be represented in several 16S rRNA clone libraries). Details about the treatments and the corresponding clone libraries are shown in Table 2.

Diversity of arrA gene sequences

The phylogenetic analysis of the translated amino acid ArrA sequences showed that the Holocene sediment (prior to incubation) was dominated by sequences closely related to environmental ArrA sequences detected previously in West Bengal arsenic-rich sediments (Héry *et al*., 2010), constituting a cluster of sequences distinct to those of any cultured organism (Fig. 5). Incubation with ^{13}C-labelled substrates led to the selection of different ArrA phylotypes. After incubation with ^{13}C-acetate, 96.7% of the ArrA sequences obtained were related to *G. uraniireducens* Rf4 and 3.2% to *Geobacter lovleyi* SZ putative ArrA sequences (δ-*Proteobacteria*) (Fig. 5). Incubation with ^{13}C-lactate selected for a distinct ArrA phylotype that was not affiliated directly to any known arsenate-respiring microorganism.

Although polymerase chain reaction (PCR) amplification of *arrA* gene fragments from the Pleistocene sediment was not successful, *arrA* gene sequences were successfully amplified after incubation with lactate for 77 days. The ArrA sequences obtained from the 'P + lactate' treatment clustered with environmental ArrA sequences from Utah basin aquifer sediments and from Chesapeake Bay sediments (Song *et al*., 2009), and they were distantly related to ArrA sequences from *Geobacter* species (Fig. 5).

Discussion

The effect of organic substrate amendments on the phylogenetic structure of indigenous bacterial communities and their potential for arsenic mobilization were investigated in two contrasting arsenic-rich Cambodian sediments: (i) a Holocene sediment, rich in labile organic matter and with a solid phase arsenic speciation dominated by As(III), and (ii) a Pleistocene sediment, with low abundances of predominantly recalcitrant organic matter and with a solid phase arsenic speciation dominated by As(V). Detailed organic matter analysis of the two sediments is provided as Supporting Information Appendix S1.

In the microcosms that were set up with the Holocene sediment, the use of ^{13}C-labelled substrates, DNA-SIP, gene cloning and sequencing of the heavy DNA fraction allowed the profiling of the active members of the bacterial communities, highlighting the applicability of DNA-SIP as a tool in microbial ecology studies, as previously

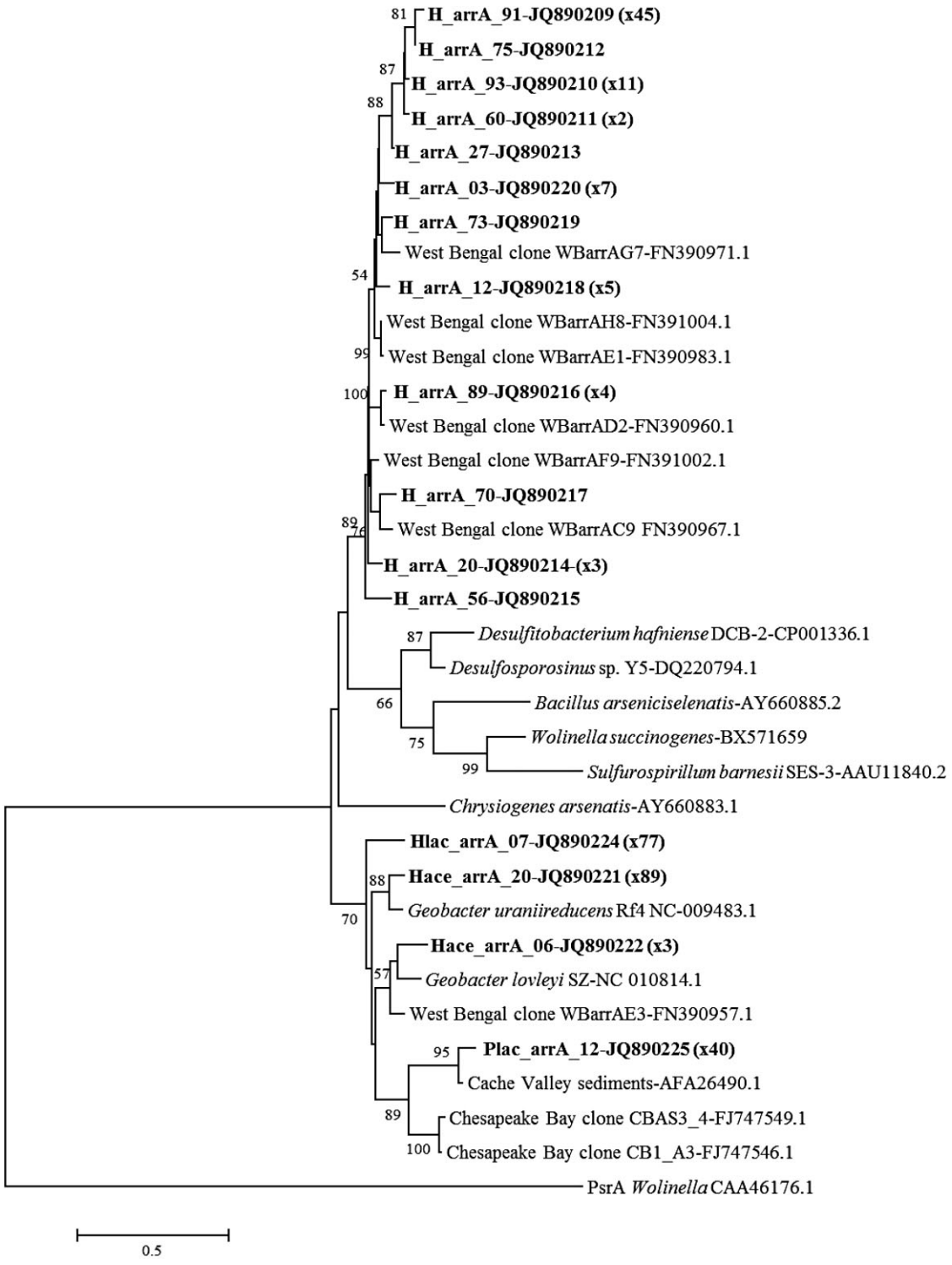

Fig. 5. ArrA phylogenetic tree based on 203 AA sequences using maximum-likehood method under Jones–Taylor–Thornton (JTT) substitution model, with a gamma distribution of rates between sites. ArrA sequences (in bold) were grouped into phylotypes based on a 98% nucleotidic sequence similarity cut-off. The number of clones contained in each phylotype is indicated in brackets. Details about the different samples are shown in Table 2.

discussed (Neufeld *et al*., 2007a). Incubation of Holocene sediments with ^{13}C-lactate and ^{13}C-acetate led to the enrichment of *Geobacter*-related 16S rRNA sequences. However, different *Geobacter*-related phylotypes were enriched after amendment with ^{13}C-lactate or ^{13}C-acetate, which may indicate organic carbon utilization specificity at the bacterial species level. Moreover, both substrates resulted in the enrichment of sequences closely related to dissimilatory arsenate-reducing *Sulfurospirillum* spp. (Stolz *et al*., 1999; Luijten *et al*., 2003). Previous studies that were carried out using acetate as the organic carbon proxy also showed enrichment in sequences belonging to the *Geobacter* (Lear *et al*., 2007; Héry *et al*., 2010) and *Sulfurospirillum* genera (Lear *et al*., 2007), and the potentially important role of the *Geobacter* genus on arsenic cycling has been previously discussed in South East Asian aquifers (Lear *et al*., 2007; Héry *et al*., 2010; Lloyd *et al*., 2011) and more recently in a uranium-contaminated aquifer (Giloteaux *et al*., 2013). However, it should be noted that further work is required to confirm that enrichment of *Geobacter* species in these sediment systems is linked directly to arsenic cycling, and not other biogeochemical processes stimulated by the addition of organic carbon.

In this study, the enrichment of potential arsenate-respiring *Geobacter* and *Sulfurospirillum* spp. and the detection of ArrA sequences in the heavy DNA fractions was not accompanied by As(III) mobilization from As(V) naturally present in the Holocene sediment during the incubation period, in contrast with previous microcosm studies (Lear *et al*., 2007; Héry *et al*., 2010). Thus, it is not clear whether naturally occurring As(V) served as the electron acceptor during the lactate/acetate utilization. The As(V) in the solid phase comprised only 7% of the total solid phase arsenic, and a significant fraction could be non-bioavailable, for example incorporated into recalcitrant mineral phases. Because *Geobacter* spp. are known Fe(III)-reducing bacteria, it is more likely that substrate utilization was mainly coupled with reduction of mineral Fe(III)-oxides present in the Holocene sediments. This is supported by the increase of soluble Fe(II) concentrations observed during similar incubations, suggesting that iron reduction occurred in the Holocene microcosms (data not shown).

The phylogenetic analysis of the bacterial community in the Pleistocene sediment (P clone library; Fig. 2) showed that it shared no common OTUs with the bacterial community in the Holocene sediment. This finding indicates that contrasting bacterial communities inhabit Pleistocene and Holocene sediments, and that these communities may have different potentials for substrate utilization and As(III) mobilization. Moreover, no known Fe(III)- or As(V)-reducing bacteria were initially detected in the Pleistocene sediment, despite the relatively diverse bacterial community present, in agreement with a previous study (Sutton *et al*., 2009), and *arrA* gene PCR amplification was not successful.

However, the addition of a simple and labile organic carbon substrate (lactate) stimulated the microbially mediated release of As(III) into the aqueous phase (0.16 μM on day 77, Fig. 1C). Incubation with lactate favoured the dominance of sequences closely related to the sulphate-reducing bacterium *D. oxamicus* DSM 1925 (López-Cortés *et al*., 2006) but not to *Geobacter* spp. and *Sulfurospirillum* spp., as found previously in the 'H + ^{13}C-lactate' treatment. The dominance of a sulphate-reducing bacterium in microcosms that were not amended with sulphate could be explained by the fact that the natural sulphate content in the Pleistocene sediment prior to incubation was 40 mg l^{-1}, but it was depleted by the 28th day of incubation (data not shown). Moreover, another *Desulfovibrio* species, *Desulfovibrio desulfuricans* G20, has been shown to reduce 20 mM As(V) in liquid cultures with lactate as the electron donor and sulphate as the electron acceptor, using a detoxification mechanism regulated by an *arsRBCC* operon and an *arsC* gene (Li and Krumholz, 2007). In the past, it had been suggested that *Desulfovibrio* spp. might be contributing to arsenate respiration in Mono Lake waters (Hoeft *et al*., 2002), but no evidence of arsenate respiratory reductase genes (*arrA* and *arrB*) has been found in the *D. desulfuricans* G20 genome (Li and Krumholz, 2007). In our study, the translated ArrA sequences that were retrieved from the 'P + lactate' treatment were only distantly related to ArrA sequences from *Geobacter* species. Thus, it is not clear whether the low As(III) mobilization in these Pleistocene microcosms was driven by a yet uncharacterized arsenate-respiring *Desulfovibrio* species or by members of the microbial community present in low abundances. Although *Geobacter* 16S rRNA gene sequences have been detected previously in Pleistocene sediments (Al Lawati *et al*., 2012), their involvement in arsenic mobilization in the Cambodian Pleistocene sediments is not supported clearly by the present study.

Future experiments with ^{13}C-labelled substrates will allow a more accurate identification of the active members of microbial communities in Pleistocene sediments, capable of dissimilatory arsenate reduction. However, our findings demonstrate clearly that Pleistocene sediments are poised ready for As reduction and mobilization, limited only by the lack of suitable organic substrates. This is consistent with Postma and colleagues (2012) who suggested that lower groundwater arsenic in similar aquifers in northern Vietnam is associated with the occurrence of older, less reactive organic matter. In the presence of an organic carbon proxy such as lactate, As(III) mobilization meditated by indigenous microorganisms can occur in Pleistocene aquifers under anoxic conditions. This

finding, in contradiction with what was previously observed for Pleistocene sediments from Bengladesh (Sutton *et al*., 2009; Dhar *et al*., 2011; Al Lawati *et al*., 2012), has major implications for future strategies that aim to reduce the exposure to arsenic by switching to consumption of groundwater from Pleistocene aquifers, particularly where there is significant advective transport of labile surface-derived organic carbon electron donors (Harvey *et al*., 2002; van Geen *et al*., 2013; Lawson *et al*., 2013; Mailloux *et al*., 2013).

Experimental procedures

Site location and sediment collection

The two sampling sites were chosen according to data available from previous studies in order to collect sediment samples that correspond to Holocene and Pleistocene aquifers. The sites near the Mekong River in Kandal Province, Cambodia, had been identified previously as a region of high arsenic groundwater hazard, with concentrations ranging from 10 to > 1000 μg l^{-1} (Polya *et al*., 2003; 2005; Berg *et al*., 2007; Buschmann *et al*., 2007; Kocar *et al*., 2008; Lado *et al*., 2008; Polizzotto *et al*., 2008; Rowland *et al*., 2008; Lawson *et al*., 2013), and where both Pleistocene and Holocene sediments are found (Tamura *et al*., 2007; 2009). According to Polizzotto and colleagues (2008), arsenic contents in the sediments in this area range between 2.8 and 12 mg kg^{-1}, whereas the average crustal abundance of arsenic is 1.5 mg kg^{-1}. The Holocene sediments typically comprise clays, peats and silts overlying fine grey sands (Tamura *et al*., 2007; Kocar *et al*., 2008). Tamura and colleagues (2009) characterized older Pleistocene deposits as orange sands and silts occasionally underlying the Holocene sediments. Two distinct sediments were selected for this study based on sampling location and information inferred about their geological history, as well as from visual observations made on the retrieved cores: one Holocene sediment (H) and one deeper sediment assumed to be 'Pleistocene' (P) on the basis of its lithological characteristics, which were consistent with Pleistocene sediments previously described in the same area by Tamura and colleagues (2009), and its occurrence below sediments of inferred Holocene age. The Holocene sediments were collected at Rotaing (Cambodia) on 11 July 2008 [Global Positioning System (GPS) positions were 48P 0503670, 1271762]. The well used for drilling fluid (water) was 63 m deep. Two cores were collected between 11 and 11.5 m depth, and the sediments were composed of a mix of clay and fine-grained grey sand. The Pleistocene sediments were collected at Sre Ampil, Chheu Teal, Kean Svay District (Cambodia) on 22 July 2008 (GPS positions were 0509998, 1266782). The well used for drilling was 31 m deep. Four cores were collected between 12 and 13.5 m depth, and the sediments were composed of fine to medium orange sand.

All sediment samples were collected using a 'core catcher' going down inside driller pipes, thereby removing the possibility of contamination from borehole sides. When the cores were collected sequentially, the borehole was flushed with groundwater between collection for 1 min (i.e. no drilling between sample collection and quick flush with groundwater). The drilling fluid (water) was always sourced from nearby anoxic groundwater well (> 20 m). All the samples were sealed into O_2-impermeable bags (with at least one O_2 stripper included) using a larger glove bag under N_2 and refrigerated within 6 h of collection. They were maintained refrigerated during transport by courier to Manchester (UK), where they were handled using strictly anoxic conditions, stored at 10°C to minimize oxidation (Rowland *et al*., 2005) and used in microcosm studies within 2 weeks.

Sediment characteristics

Solid-phase organic matter characterization, including TOC and TN determinations and lipid biomarker characterization, was performed as previously described in Héry and colleagues (2010). TC and TN values were determined using a EuroVector EA3000 CHN elemental analyser (EuroVector, Milan, Italy). The inorganic carbon content was determined using a modified Strohlein Coulomat 702 analyser (Markus de Vries, Schwabach, Germany), and TOC was calculated by subtracting inorganic carbon from TC values. Experimental procedures related to organic matter analyses are provided as Supporting Information Appendix S1.

Microcosm set up

Microcosms were set up as described in Héry and colleagues (2010); 10 g of anoxic sediments were placed into sterile acid washed 100 ml glass serum vials and mixed with 20 ml of sterile synthetic groundwater (Rowland *et al*., 2007). The microcosms were set up under an N_2 atmosphere, sealed with rubber butyl stoppers, then flushed with a mixture of CO_2 : N_2 (ratio 20:80) for 10 min, and incubated at 20°C. With the Holocene sediment, two treatments were set up (Table 2), as microcosms were amended with 10 mM ^{13}C-lactate or 10 mM ^{13}C-acetate. The two treatments, including additional abiotic controls (sterilized by autoclaving), were set up in triplicate. To validate the further separation of ^{12}C- and ^{13}C-DNA, controls were conducted by incubating bacterial pure cultures with either ^{12}C- or ^{13}C-acetatem and the substrate consumption was checked over time (data not shown). With the Pleistocene sediment, the microcosms were amended with (i) 10 mM unlabelled lactate or (ii) 10 mM unlabelled acetate. Because no acetate utilization was observed during 100 days of incubation, only results from the 10 mM lactate microcosms ('P + lactate') are presented (Table 2).

Organic acid quantification and arsenic speciation

Subsamples were collected at different incubation times for analyses (Fig. 1), and the solid phase was separated from aqueous phase as described previously (Héry *et al*., 2010). The concentrations of lactate and acetate (^{13}C-labelled or unlabelled) in the microcosm supernatants and in the control cultures were determined using a Dionex DX120 ion chromatograph, fitted with a Dionex ICE AS1 ion-exclusion column (Dionex, Sunnyvale, CA, USA). When most of the lactate or acetate was consumed in the amended microcosms (Table 2; Fig. 1) and in the pure cultures, the incubations were stopped and the corresponding samples were stored at −80°C for further analyses.

Table 2. The experimental treatments of this study, the incubation periods, and the corresponding 16S rRNA gene or *arrA* gene clone libraries (including GenBank accession numbers).

Treatment	Incubation period (d)	DNA fraction	16S rRNA gene clone library	Accession numbers	arrA clone library	Accession numbers
Holocene sediment	–	Unlabelled	H	JQ810187-JQ810259	H_arrA	JQ890209-JQ890220
H + ^{13}C-lactate	7	^{13}C	Hlac	JQ810260-JQ810332	Hlac_arrA	JQ890224
H + ^{13}C-acetate	43	^{13}C	Hace	JQ810495-JQ810568	Hace_arrA	JQ890221-JQ890222
Pleistocene sediment	–	Unlabelled	P	JQ810569-JQ810650	–	–
P + lactate	77	Unlabelled	Plac	JQ810651-JQ810718	Plac_arrA	JQ890225
P + acetate	100[a]	–	–	–	–	–

a. No acetate degradation was observed during the 100-days incubation period.

Arsenic speciation in the aqueous phase was determined by Ion Chromatography Inductively Coupled Plasma Mass Spectrometry, as described previously (Héry *et al*., 2010). Arsenic speciation in the sediments was determined from XANES spectra collected on the I18 beamline at the DIAMOND synchrotron (Didcot, UK) using linear combination analysis on the underivatized normalized spectra, in comparison with model compounds, sodium arsenate (As(V)-O), sodium arsenite (As(III)-O) and arsenopyrite (As-S).

DNA isolation and stable isotope probing

DNA was extracted from all the Holocene and Pleistocene sediment samples as well as from the control cultures using the PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA). After DNA extraction from the Holocene microcosms amended with ^{13}C-lactate and ^{13}C-acetate (Table 2), and from the control cultures, heavy and light DNA fractions were separated on a CsCl gradient according to the protocol by Neufeld and colleagues (2007b), adapted for 3.9 ml Quick-Seal®, Polyallomer tubes (Beckman Coulter, Brea, CA, USA). The ^{12}C- and ^{13}C-control DNA were run in parallel to Holocene DNA samples to confirm that the DNA recovered in the heavy fraction corresponds to organisms that had actively metabolized the substrate. Following centrifugation with an Optima TL-100 ultra centrifuge (Beckman Coulter) at 178 693 *g* for 47 h at 20°C (maximum acceleration, no deceleration), 11 fractions of approximately 330 µl were collected from each sample, using a low-flow rate peristaltic pump, and their density was determined using an analytical balance (for each sample and the control tube). The DNA in the collected fractions was precipitated as described previously (Neufeld *et al*., 2007b), resuspended in 30 µl of Tris-EDTA buffer and quantified using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The recovered DNA fractions were used as templates for ribosomal intergenic spacer analysis , using primers ITSF and ITSReub (Cardinale *et al*., 2004), in order to verify that they contained distinct bacterial populations corresponding to successful ^{12}C- and ^{13}C-DNA separation along the density gradient (data not shown). In addition, 16S rRNA-based DGGE was performed on a selection of DNA fractions from the H + ^{13}C-lactate and H + ^{13}C-acetate treatments in order to demonstrate the distinct DGGE patterns among the (i) heavy DNA fraction, (ii) light DNA fraction and the (iii) total DNA (before separation on the CsCl density gradient). PCR amplifications were carried out using GC-341F and 518R (Muyzer *et al*., 1993) and touchdown PCR protocol (Cunliffe and Kertesz, 2006). 16S rRNA gene PCR products were purified through a QIAquick PCR purification column (QIAGEN, Valencia, CA, USA) and quantified with a Nano-drop spectrophotometer (Nanodrop, Wilmington, DE, USA). DGGE was carried out in a DCode electrophoresis chamber (Bio-Rad, Hercules, CA, USA) as described previously (Cunliffe and Kertesz, 2006). Gels were stained with SYBR Gold nucleic acid stain (Invitrogen, Carlsbad, CA, USA) and viewed under an UVItec trans-illuminator (UVitec, Cambridge, UK). Selected DGGE bands were excised from gel using a scalpel blade, crushed in 20 µl of dH_2O and incubated for 48 h at 4°C. The released DNA was then re-amplified as above but using the unclamped 341F primer. 16S rRNA gene PCR products were purified as above and used for sequencing. The sequences obtained were checked for chimeras using Pintail (Ashelford *et al*., 2005), and their closest phylogenetic affiliation (to cultured organism, if possible) was identified by nucleotide Blastn searches.

16S rRNA and arrA gene cloning and sequencing

PCR amplification of isolated DNA (unlabelled or selected heavy DNA fractions, as shown in Table 2), cloning and sequencing of 16S rRNA gene and *arrA* gene fragments were performed as described previously (Héry *et al*., 2010). To account for *arrA* gene variability, we tested multiple sets of available primers to cover maximal *arrA* diversity. All *arrA* primers and PCR amplification protocols published to date were tested (Malasarn *et al*., 2004; Kulp *et al*., 2006; Lear *et al*., 2007; Fisher *et al*., 2008). None resulted in amplification of *arrA* sequences from DNA isolated from the Holocene and Pleistocene sediments. The only protocol that successfully amplify an *arrA* gene product was the one described previously by Héry and colleagues (2010). First, *arrA* was amplified using primers ArrAUF1 (TGTCAAGGHTGTA CBDCHTGG) and ArrAUR3 (GCW GCCCAY TCV GGN GT) as described by (Fisher *et al*., 2008). A second PCR amplification was performed with primers AS2F (GTCCCNA TBASNTGGGANRARGCNMT) and AS1R (GGGGTGCG GTCYTTNARYTC) as described by Lear and colleagues (2007), using the first PCR products as templates. In this study, five 16S rRNA and four *arrA* gene clone libraries were prepared (Table 2); for each library, either 96 or 48 of clones were sequenced. The 16S rRNA gene sequences obtained were analysed for chimeras using Mallard (Ashelford *et al*.,

2006), and chimeric sequences were removed from further analysis. The phylogenetic classification of the non-chimeric 16S rRNA sequences (at a confidence threshold of 95%) was performed using the RDP Naive Bayesian rRNA Classifier Version 2.4 of the Ribosomal Database Project (Cole *et al*., 2009), and MOTHUR v.1.6.0 (Schloss *et al*., 2009) was used to cluster these sequences into OTUs at a level of similarity of 97%. The closest type strain relative to each OTU was identified using the RDP(Ribosomal Database Project)'s SeqMatch. The phylogenetic tree of the *Geobacter*-related 16S rRNA sequences was drawn with MEGA5 (Tamura *et al*., 2011), using the neighbour-joining method and the Jukes Cantor substitution model. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. The *arrA* sequences obtained were submitted to BLAST search (Altschul *et al*., 1990), and phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura *et al*., 2011). The best-fitting model of molecular evolution for the dataset was calculated based on amino acid-derived sequences, and the optimal tree was performed with maximum-likehood method under Jones–Taylor–Thornton substitution model, with a gamma distribution of rates between sites. Nodal robustness of the tree was assessed using 1000 bootstrap replicates.

All sequences of this study were deposited to GenBank, under accession numbers JQ810187-JQ810718 (16S rRNA gene sequences; Table 2) and JQ890209-JQ890225 (*arrA* gene sequences; Table 2).

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References

Acharyya, S.K., Lahiri, S., Raymahashay, B.C., and Bhowmik, A. (2000) Arsenic toxicity of groundwater in parts of the Bengal basin in India and Bangladesh: the role of Quaternary stratigraphy and Holocene sea-level fluctuation. *Environ Geol* **39:** 1127–1137.

Al Lawati, W.M., Rizoulis, A., Eiche, E., Boothman, C., Polya, D.A., Lloyd, J.R., *et al*. (2012) Characterisation of organic matter and microbial communities in contrasting arsenic-rich Holocene and arsenic-poor Pleistocene aquifers, Red River Delta, Vietnam. *Appl Geochem* **27:** 315–325.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* **71:** 7724–7736.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72:** 5734–5741.

Berg, M., Tran, H.C., Nguyen, T.C., Pham, P.V., Schertenleib, R., and Giger, W. (2001) Arsenic contamination of groundwater and drinking water in Vietnam: A human health threat. *Environ Sci Technol* **35:** 2621–2626.

Berg, M., Stengel, C., Trang, P.T.K., Viet, P.H., Sampson, M.L., Leng, M., *et al*. (2007) Magnitude of arsenic pollution in the Mekong and Red River Deltas – Cambodia and Vietnam. *Sci Total Environ* **372:** 413–425.

Burgess, W.G., Hoque, M.A., Michael, H.A., Voss, C.I., Breit, G.N., and Ahmed, K.M. (2010) Vulnerability of deep groundwater in the Bengal Aquifer System to contamination by arsenic. *Nat Geosci* **3:** 83–87.

Buschmann, J., Berg, M., Stengel, C., and Sampson, M.L. (2007) Arsenic and manganese contamination of drinking water resources in Cambodia: coincidence of risk areas with low relief topography. *Environ Sci Technol* **41:** 2146–2152.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., *et al*. (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol* **70:** 6147–6156.

Charlet, L., and Polya, D.A. (2006) Arsenic hazard in shallow reducing groundwaters in southern Asia. *Elements* **2:** 91–96.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., *et al*. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37:** D141–D145.

Cunliffe, M., and Kertesz, M.A. (2006) Effect of *Sphingobium yanoikuyae* B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environ Pollut* **144:** 228–237.

Dhar, R.K., Zheng, Y., Saltikov, C.W., Radloff, K.A., Mailloux, B.J., Ahmed, K.M., *et al*. (2011) Microbes enhance mobility of arsenic in Pleistocene aquifer sand from Bangladesh. *Environ Sci Technol* **45:** 2648–2654.

Fisher, E., Dawson, A.M., Polshyna, G., Lisak, J., Crable, B., Perera, E., *et al*. (2008) Transformation of inorganic and organic arsenic by *Alkaliphilus oremlandii* sp nov strainOhILAs. *Ann N Y Acad Sci* **1125:** 230–241.

Gault, A.G., Islam, F.S., Polya, D.A., Charnock, J.M., Boothman, C., and Lloyd, J.R. (2005) Microcosm depth profiles of arsenic release in an arsenic contaminated aquifer, West Bengal. *Mineral Mag* **69:** 855–863.

van Geen, A., Ahmed, K.M., Seddique, A.A., and Shamsudduha, M. (2003) Community wells to mitigate the

arsenic crisis in Bangladesh. *Bull World Health Organ* **81:** 632–638.

van Geen, A., Rose, J., Thoral, S., Garnier, J., Zheng, Y., and Bottero, J. (2004) Decoupling of As and Fe release to Bangladesh groundwater under reducing conditions. Part II: evidence from sediment incubations. *Geochim Cosmochim Acta* **68:** 3475–3486.

van Geen, A., Bostick, B.C., Trang, P.T.K., Lan, Y.M., Mai, N.N., Manh, P.D., *et al*. (2013) Retardation of arsenic transport through a Pleistocene aquifer. *Nature* **501:** 204–207.

Giloteaux, L., Holmes, D.E., Williams, K.H., Wrighton, K.C., Wilkins, M.J., Montgomery, A.P., *et al*. (2013) Characterization and transcription of arsenic respiration and resistance genes during in situ uranium bioremediation. *ISME J* **7:** 370–383.

Harvey, C.F., Swartz, C.H., Badruzzaman, A.B.M., Keon-Blute, N., Yu, W., Ali, M.A., *et al*. (2002) Arsenic mobility and groundwater extraction in Bangladesh. *Science* **298:** 1602–1606.

Héry, M., van Dongen, B.E., Gill, F., Mondal, D., Vaughan, D.J., Pancost, R.D., *et al*. (2010) Arsenic release and attenuation in low organic carbon aquifer sediments from West Bengal. *Geobiology* **8:** 155–168.

Hoeft, S.E., Lucas, F., Hollibaugh, J.T., and Oremland, R.S. (2002) Characterization of microbial arsenate reduction in the anoxic bottom waters of Mono Lake, California. *Geomicrobiol J* **19:** 23–40.

Islam, F.S., Gault, A.G., Boothman, C., Polya, D.A., Charnock, J.M., Chatterjee, D., *et al*. (2004) Role of metal-reducing bacteria in arsenic release from Bengal delta sediments. *Nature* **430:** 68–71.

Kocar, B.D., Polizzotto, M.L., Benner, S.G., Ying, S.C., Ung, M., Ouch, K., *et al*. (2008) Integrated biogeochemical and hydrologic processes driving arsenic release from shallow sediments to groundwaters of the Mekong delta. *Appl Geochem* **23:** 3059–3071.

Kulp, T.R., Hoeft, S.E., Miller, L.G., Saltikov, C., Murphy, J.N., Han, S., *et al*. (2006) Dissimilatory arsenate and sulfate reduction in sediments of two hypersaline, arsenic-rich soda lakes: Mono and Searles lakes, California. *Appl Environ Microbiol* **72:** 6514–6526.

Lado, L.R., Polya, D., Winkel, L., Berg, M., and Hegan, A. (2008) Modelling arsenic hazard in Cambodia: a geostatistical approach using ancillary data. *Appl Geochem* **23:** 3010–3018.

Lawson, M., Polya, D.A., Boyce, A.J., Bryant, C., Mondal, D., Shantz, A., *et al*. (2013) Pond-derived organic carbon driving changes in arsenic hazard found in Asian groundwaters. *Environ Sci Technol* **47:** 7085–7094.

Lear, G., Song, B., Gault, A.G., Polya, D.A., and Lloyd, J.R. (2007) Molecular analysis of arsenate-reducing bacteria within Cambodian sediments following amendment with acetate. *Appl Environ Microbiol* **73:** 1041–1048.

Li, X., and Krumholz, L.R. (2007) Regulation of arsenate resistance in *Desulfovibrio desulfuricans* G20 by an *arsRBCC* operon and an *arsC* gene. *J Bacteriol* **189:** 3705–3711.

Lloyd, J.R., Gault, A.G., Héry, M., and MacRae, J.D. (2011) Microbial transformations of arsenic in the subsurface. In *Environmental Microbe-Metal Interactions II*. Stolz, J.F., and Oremland, R.S. (eds). Washington, DC, USA: ASM Press, pp. 77–90.

López-Cortés, A., Fardeau, M.-L., Fauque, G., Joulian, C., and Ollivier, B. (2006) Reclassification of the sulfate- and nitrate-reducing bacterium *Desulfovibrio vulgaris* subsp. *oxamicus* as *Desulfovibrio oxamicus* sp. nov., comb. nov. *Int J Syst Evol Microbiol* **56:** 1495–1499.

Luijten, M.L.G.C., de Weert, J., Smidt, H., Boschker, H.T.S., de Vos, W.M., Schraa, G., *et al*. (2003) Description of S*ulfurospirillum halorespirans* sp nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. *Int J Syst Evol Microbiol* **53:** 787–793.

Mailloux, B.J., Trembath-Reichert, E., Cheung, J., Watson, M., Stute, M., Freyer, G.A., *et al*. (2013) Advection of surface-derived organic carbon fuels microbial reduction in Bangladesh groundwater. *Proc Natl Acad Sci USA* **110:** 5331–5335.

Malasarn, D., Saltikov, C.W., Campbell, K.M., Santini, J.M., Hering, J.G., and Newman, D.K. (2004) *arrA* is a reliable marker for As(V) respiration. *Science* **306:** 455.

Muyzer, G., Dewaal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59:** 695–700.

Neufeld, J.D., Wagner, M., and Murrell, J.C. (2007a) Who eats what, where and when? Isotope-labelling experiments are coming of age. *ISME J* **1:** 103–110.

Neufeld, J.D., Vohra, J., Dumont, M.G., Lueders, T., Manefield, M., Friedrich, M.W., *et al*. (2007b) DNA stable-isotope probing. *Nat Protoc* **2:** 860–866.

Oremland, R.S., and Stolz, J.F. (2005) Arsenic, microbes and contaminated aquifers. *Trends Microbiol* **13:** 45–49.

Polizzotto, M.L., Kocar, B.D., Benner, S.G., Sampson, M., and Fendorf, S. (2008) Near-surface wetland sediments as a source of arsenic release to ground water in Asia. *Nature* **454:** 505–U505.

Polya, D.A., and Charlet, L. (2009) Rising arsenic risk? *Nat Geosci* **2:** 383–384.

Polya, D.A., Gault, A.G., Bourne, N.J., Lythgoe, P.R., and Cooke, D.A. (2003) Coupled HPLC-ICP-MS analysis indicates highly hazardous concentrations of dissolved arsenic species in Cambodian groundwaters. In *Plasma Source Mass Spectrometry: Applications and Emerging Technologies*. Holland, J.G., and Tanner, S.D. (eds). Cambridge: The Royal Society of Chemistry, pp. 127–140.

Polya, D.A., Gault, A.G., Diebe, N., Feldman, P., Rosenboom, J.W., Gilligan, E., *et al*. (2005) Arsenic hazard in shallow Cambodian groundwaters. *Mineral Mag* **69:** 807–823.

Postma, D., Larsen, F., Thai, N.T., Pham, T.K.T., Jakobsen, R., Nhan, P.Q., *et al*. (2012) Groundwater arsenic concentrations in Vietnam controlled by sediment age. *Nat Geosci* **5:** 656–661.

Ravenscroft, P., Burgess, W.G., Ahmed, K.M., Burren, M., and Perrin, J. (2005) Arsenic in groundwater of the Bengal Basin, Bangladesh: distribution, field relations, and hydrogeological setting. *Hydrogeol J* **13:** 727–751.

Ravenscroft, P., Brammer, H., and Richards, K. (2009) *Arsenic Pollution*, 1st edn. Oxford, UK: Wiley-Blackwell.

Rowland, H.A.L., Gault, A.G., Charnock, J.M., and Polya, D.A. (2005) Preservation and XANES determination of the oxidation state of solid-phase arsenic in shallow sedimentary aquifers in Bengal and Cambodia. *Mineral Mag* **69:** 825–839.

Rowland, H.A.L., Pederick, R.L., Polya, D.A., Pancost, R.D., Van Dongen, B.E., Gault, A.G., *et al*. (2007) The control of organic matter on microbially mediated iron reduction and arsenic release in shallow alluvial aquifers, Cambodia. *Geobiology* **5:** 281–292.

Rowland, H.A.L., Gault, A.G., Lythgoe, P., and Polya, D.A. (2008) Geochemistry of aquifer sediments and arsenic-rich groundwaters from Kandal Province, Cambodia. *Appl Geochem* **23:** 3029–3046.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., *et al*. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537–7541.

Smith, A.H., Lingas, E.O., and Rahman, M. (2000) Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull World Health Organ* **78:** 1093–1103.

Song, B., Chyun, E., Jaffé, P.R., and Ward, B.B. (2009) Molecular methods to detect and monitor dissimilatory arsenate-respiring bacteria (DARB) in sediments. *FEMS Microbiol Ecol* **68:** 108–117.

Stolz, J.F., Ellis, D.J., Blum, J.S., Ahmann, D., Lovley, D.R., and Oremland, R.S. (1999) *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the ε Proteobacteria. *Int J Syst Bacteriol* **49:** 1177–1180.

Sutton, N.B., van der Kraan, G.M., van Loosdrecht, M.C.M., Muyzer, G., Bruining, J., and Schotting, R.J. (2009) Characterization of geochemical constituents and bacterial populations associated with As mobilization in deep and shallow tube wells in Bangladesh. *Water Res* **43:** 1720–1730.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731–2739.

Tamura, T., Saito, Y., Sieng, S., Ben, B., Kong, M., Choup, S., *et al*. (2007) Depositional facies and radiocarbon ages of a drill core from the Mekong River lowland near Phnom Penh, Cambodia: Evidence for tidal sedimentation at the time of Holocene maximum flooding. *J Asian Earth Sci* **29:** 585–592.

Tamura, T., Saito, Y., Sieng, S., Ben, B., Kong, M., Sim, I., *et al*. (2009) Initiation of the Mekong River delta at 8 ka: evidence from the sedimentary succession in the Cambodian lowland. *Quat Sci Rev* **28:** 327–344.

Uhlík, O., Jecná, K., Leigh, M.B., Macková, M., and Macek, T. (2009) DNA-based stable isotope probing: a link between community structure and function. *Sci Total Environ* **407:** 3611–3619.

Zheng, Y., van Geen, A., Stute, M., Dhar, R., Mo, Z., Cheng, Z., *et al*. (2005) Geochemical and hydrogeological contrasts between shallow and deeper aquifers in two villages of Araihazar, Bangladesh: implications for deeper aquifers as drinking water sources. *Geochim Cosmochim Acta* **69:** 5203–5218.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Organic matter analysis in Holocene and Pleistocene sediments.